Supplementary Information

Liu T *et al*. Expression of the fetal hematopoiesis regulator FEV indicates leukemias of prenatal origin.

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Supplementary Figures S1-8

Supplementary Methods

Isolation of hematopoietic stem and progenitor cells from human umbilical cord blood

Lin^- or CD34-enriched cells were achieved by Ficoll gradient centrifugation and magnetic-bead separation as previously described. [1, 2] Lin^- or CD34-enriched cells were stained with lineage-APC, CD34-PE-cy7, and CD38-PE (BD Bioscience) at 4 °C for 30 min in PBS containing 0.5% bovine serum albumin (BSA; Sigma). Subsequently, $Lin^-CD34^+CD38^-$ and $CD38^+$ cells were flow-sorted by using a flow cytometer (Aria II; BD).

RT-qPCR detection of FEV-expression

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions (Invitrogen) and reversely transcribed. RT-qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. All experiments were performed in triplicate with Applied Biosystems 7900HT. PCR reaction was conducted as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min. And 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s for dissociation curve. Differences in cDNA input were normalized by GUS expression levels. Primer sequences are summarized in Supplementary Table S4.

Immunophenotypic analysis of BM cells of mouse recipients

BM cells of recipients were flushed down with Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 1% BSA (Sigma). Afterward, the cells were treated with ACK solution (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, reagents from Sigma) at room temperature for 5–10 min to lyse red blood cells. The cells were immediately washed and then resuspended in PBS supplemented with 0.5% BSA for antibody staining at 4 °C for 30 min in the dark: CD45-APC or CD45-PE-Cy7 for engraftment; CD34-PE-Cy7 for total stem/progenitor cells; and CD34-PE-Cy7 and CD38-PE for engrafted HSCs. Stained cells were analyzed or sorted using flow cytometer (Aria II; BD). All antibodies were obtained from BD Bioscience.

Cell cycle analysis

Cord blood $Lin^-CD34^+CD38^-$ cells transduced with Ctr.V or iFEV were cultured in stem cell-maintaining medium for 3 days. Cells were collected, fixed and permeablized according to the manufacturer's instructions (BD Bioscience). Subsequently, the cells were stained with ki-67-PE and DAPI. Cells were analyzed using flow cytometry (LSRFortessa II; BD).

Cell apoptosis analysis

Cord blood $Lin^-CD34^+CD38^-$ cells transduced with Ctr.V or iFEV were cultured in stem cell maintaining medium for 3 days. The cell viability was determined by flow cytometry staining with annexin-V and propididium iodide (BD Bioscience) according to the manufacturer's instructions.

Generation of *fev*:GFP transgenic line

We obtained a fish line through a large-scale Tol2 transposon-mediated enhancer-trap screen (unpublished data). Sequence analysis of the insertion site by linker-mediated PCR revealed that this line trapped gene *fev* at approximately 400 base pairs (bp) upstream of its putative transcription start site.

Whole mount in situ hybridization

Whole mount in situ hybridization with zebrafish embryos was performed using a ZF-A4 in situ hybridization machine (Zfand, China) with *fev* probes as previously described[3].

Supplemental References

1. Gupta R, Hong D, Iborra F, Sarno S, Enver T. NOV (CCN3) functions as a regulator of human hematopoietic stem or progenitor cells. *Science* 2007; **316:** 590-593.
2. Hong D, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S, *et al.* Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. *Science* 2008; **319:** 336-339.
3. Wang L, Liu T, Xu L, Gao Y, Wei Y, Duan C, *et al.* Fev regulates hematopoietic stem cell development via ERK signaling. *Blood* 2013; **122:** 367-375.

Supplemental Tables (See excel files)

Table S1. Clinical information of infant leukemia patients and FEV positivity

Table S2. Clinical information of adult leukemia patients and FEV positivity

Table S3. Clinical information of child, teenager and young adult leukemia patients and FEV positivity

Table S4. Primers for RT-qPCR

Supplementary Figure Legends

Supplementary Figure S1. *fev*-expression profile in zebrafish hematopoietic cells. (**a**) Whole-mount in situ hybridization (WISH) showed that *fev* was expressed in the lateral plate mesoderm at 10-somite stage (left panel, arrowheads) and aorta-gonad-mesonephros (AGM) at 24 hpf (middle panel, arrowhead) but not in the kidney marrow at 4 dpf (right panel, dashed circle). (**b**) In the transgenic line using the *fev* promoter (*fev*:GFP), GFP fluorescence was observed in the AGM region at 24 hpf (left panel) and in pancreas but not in the adult kidney of adult zebrafish at 3 months (middle and right panels).

Supplementary Figure S2. FEV-expression profile in mouse hematopoietic cells in development. (**a**) Flow-sorting and purity detection of lineage-negative (Lin^-) and lineage-positive (Lin^+) cells of mouse 14.5 dpf fetal liver (FL), 1–4 weeks (wks) and 8 wks old bone marrow (BM). (**b**) Representative amplification and dissociation curves of Fev and Gus in the mouse cells labeled. (**c**) The results of all the samples tested are

summarized. Only FL Lin^- cells are positive in Fev-expression. Four independent experiments were conducted.

Supplementary Figure S3. Flow-sorting and FEV-expression of human hematopoietic stem and progenitor cells. (**a**) $CD34^+$ and $CD34^-$ hematopoietic cells (expressing CD45) from the tissues labelled were flow-sorted with high purities. (**b**) The RT-qPCR products of $CD34^+$ cells were cloned and sequencing confirmed FEV. (**c**) Flow-sorting and purity detection of $Lin^-CD34^+CD38^-$ and $Lin^-CD34^+CD38^+$ fractions from CD34-enriched CB cells. (**d**) RT-PCR analysis of FEV-expression in the cell fractions of **c**.

Supplementary Figure S4. iFEV effect on apoptosis and cell cyle of CB HSCs. (**a** and **b**) Apoptotic analysis in the transduced cells by representative flow-cytometric plots (**a**) and statistical summary (**b**). (**c** and **d**) Cell cycle in the in the transduced cells by representative flow-cytometric plots (**c**) and statistical summary (**d**). n = 3 separate experiments. $^{\#}P$ >0.05 (Student's t test). All Data are presented as mean ± SD.

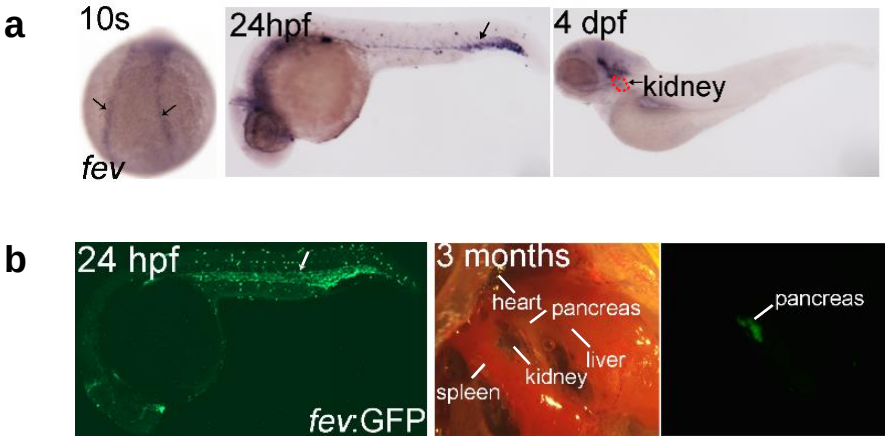
Supplementary Figure S5. RT-qPCR detection of the expression of the genes labeled in the cells flow-sorted in Figure 2b. Expression of MLL, SCL, IKZF1, BMI-1, and MEIS1 was significantly reduced in iFEV cells. n = 4 separate experiments. *P < 0.05, **P < 0.01 (student's t test). All Data are presented as mean ± SD.

Supplementary Figure S6. The correlation of FEV-expression levels with leukemia subtypes and age in FEV^+ pediatric samples. (**a**) Analysis of correlation of FEV-expression levels with leukemia subtypes. $*P < 0.001$ (Spearman's rank correlation). (**b**) Analysis of correlation of FEV-expression levels with age in leukemic samples. $^{\#}P > 0.05$ (Pearson correlation).

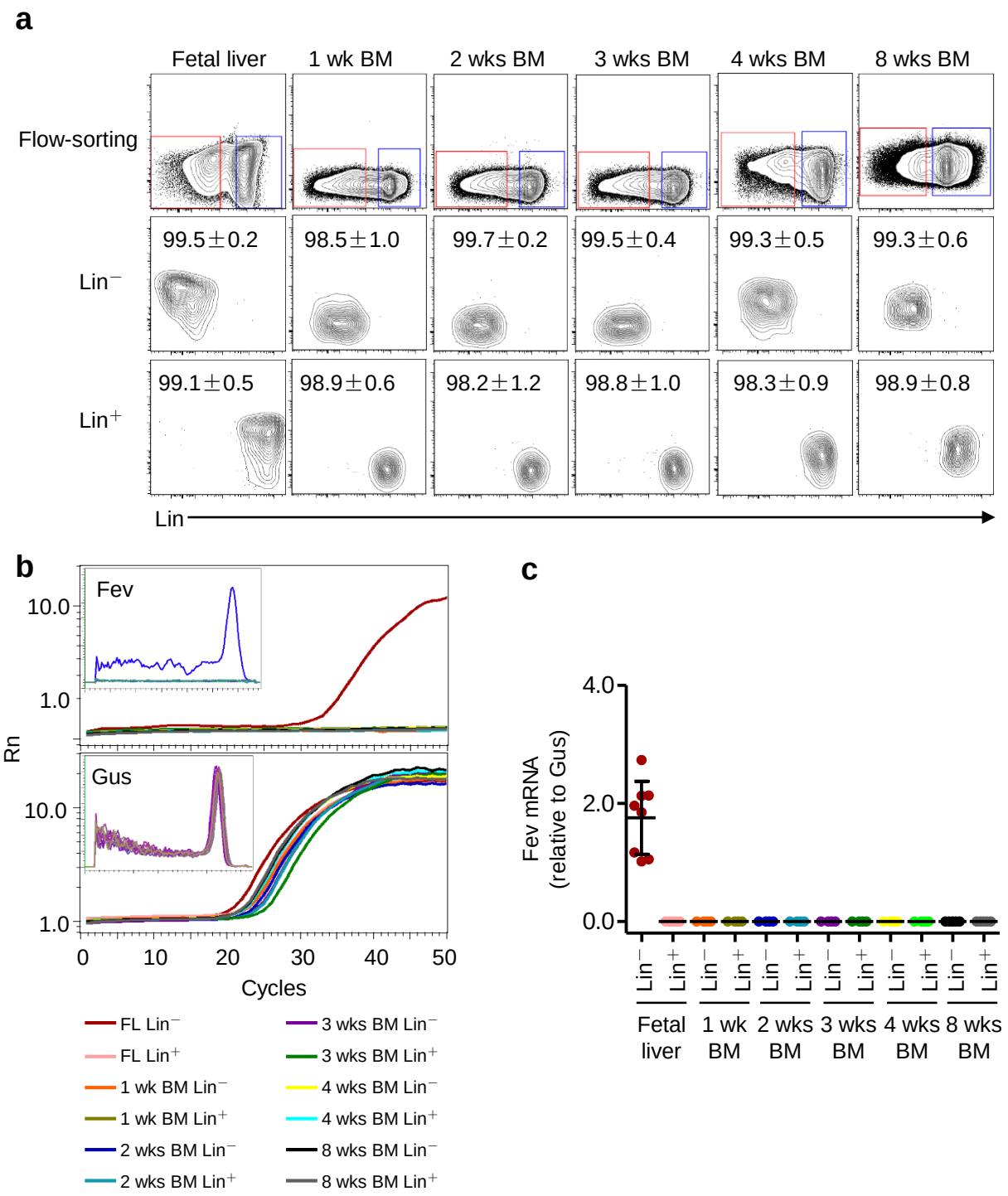
Supplementary Figure S7. Flow-sorting of AML LSCs and FEV-expression in the transduced LSCs. (**a**) Flow cytometric analysis, sorting and purity detection of LMPP- and GMP-like LSCs from FEV^+ AML cells. (**b**) Flow cytometric analysis of infection efficiencies of LMPP- and GMP-like LSCs by iFEV or Ctr.V virus. GFP^+ cells were flow-sorted. (**c**) RT-PCR analysis of FEV-expression in the flow-sorted cells of **b**. PC, positive control using 293T cells permanently expressing human FEV. NC, non-template control.

Supplementary Figure S8. Effect of iFEV on the reconstitution of human BM HSCs in xenograft mice. (**a**) Flow-cytometric analysis of engraftment and contribution of GFP^+ cells in recipients. (**b**) Statistical summary of infection efficiency before transplantation (input) and contribution of GFP^+ cells in recipients (output). $n = 6$ mice in 3 separate experiments. $^{\#}P > 0.05$ (Student's t test). All Data are presented as mean ± SD.

Supplementary Figure S1



Supplementary Figure S2



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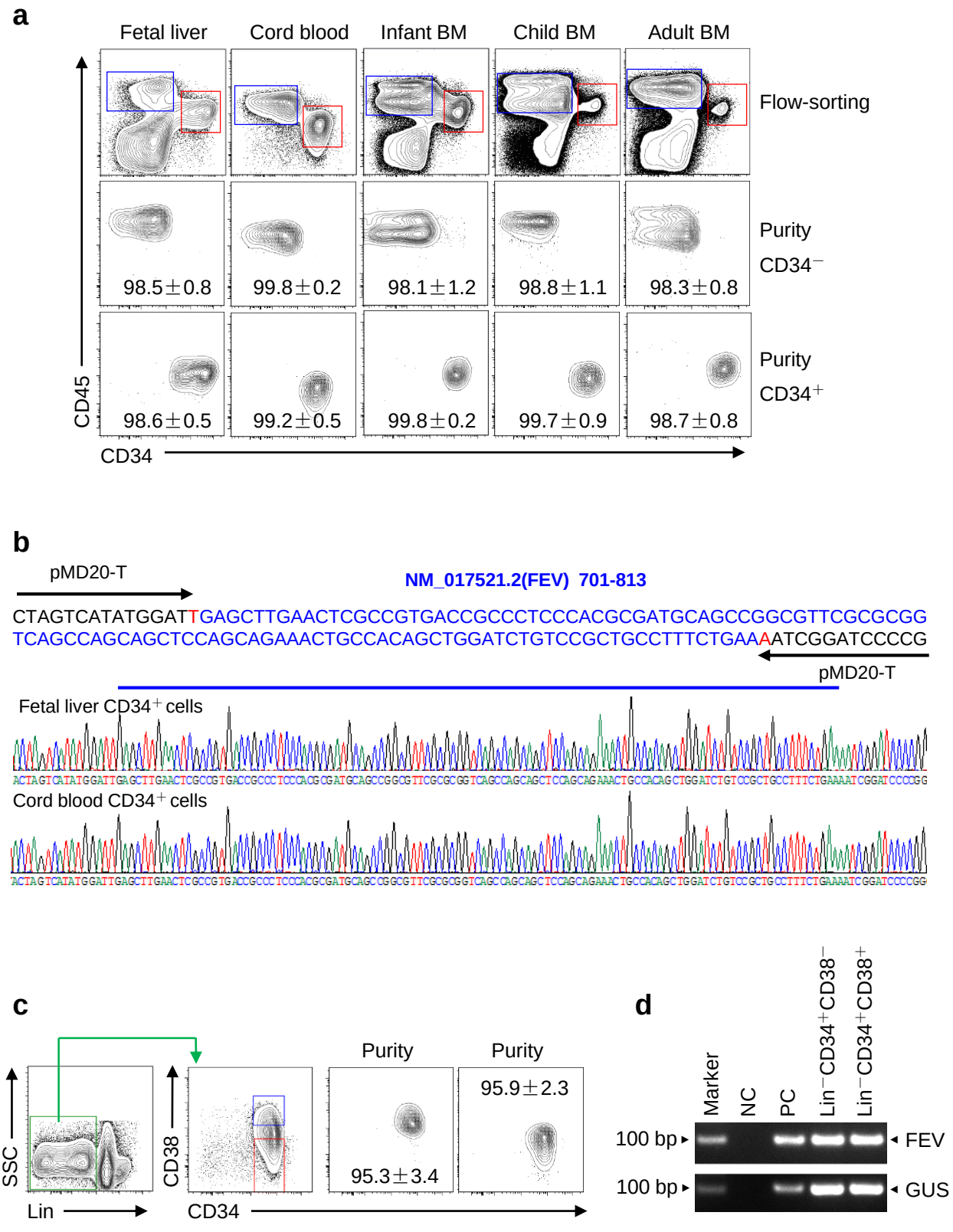

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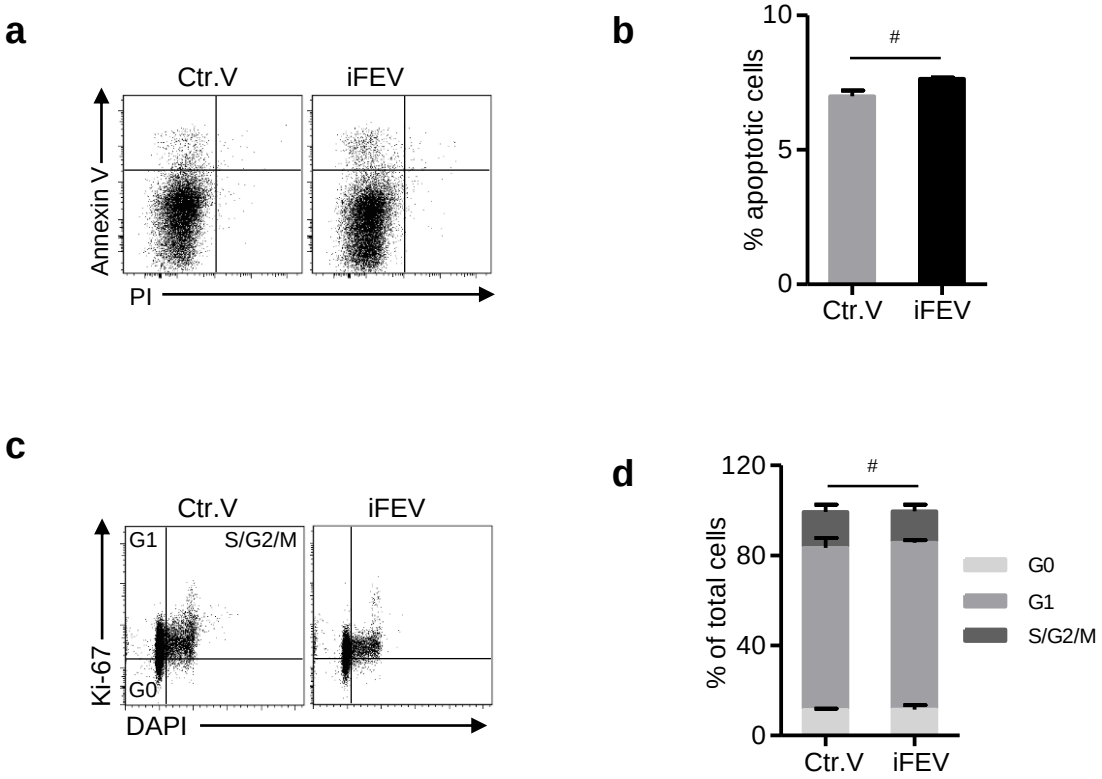

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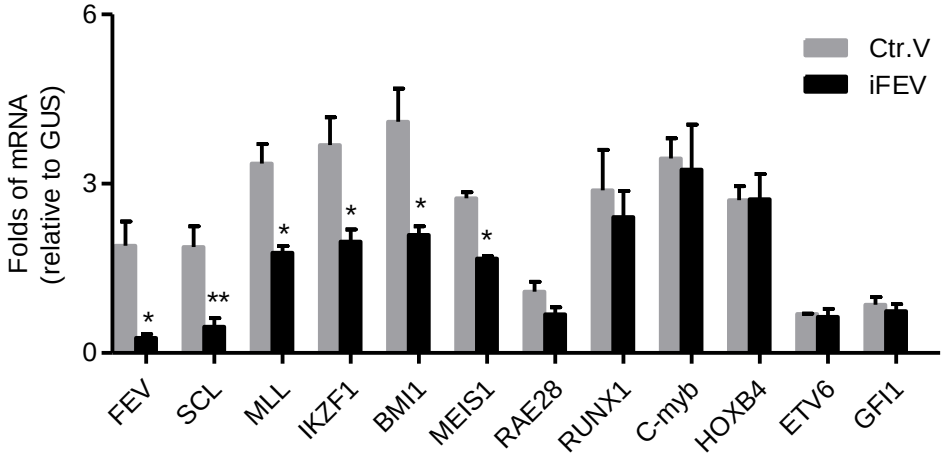

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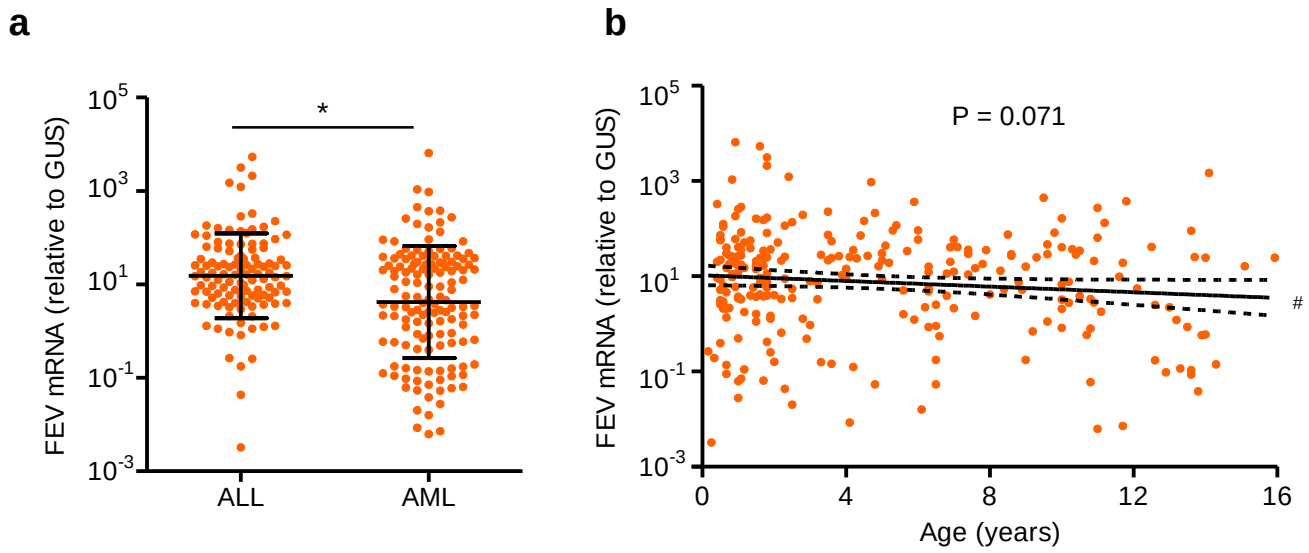
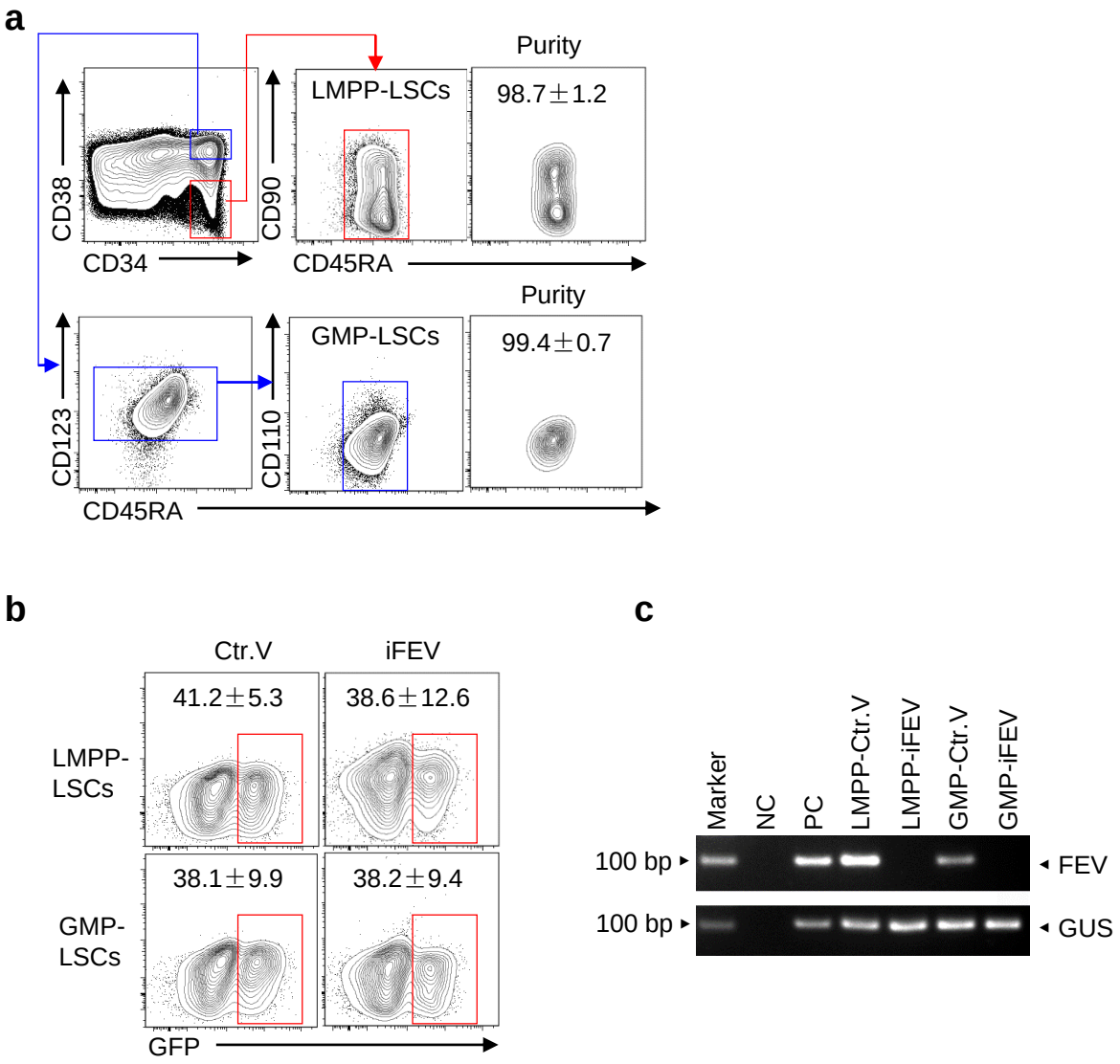


Supplementary Figure S7



Supplementary Figure S8